J. Dairy Sci. 104:9801–9812
https://doi.org/10.3168/jds.2020-20075

Effect of protein concentrate mixtures and dietary addition of exogenous phytase on major milk minerals and proteins, including casein phosphorylation

N. A. Poulsen,[1]* G. Giagnoni,[2] M. Johansen,[2] P. Lund,[2] and L. B. Larsen[1]
[1]Department of Food Science, Aarhus University, Agro Food Park 48, DK-8200 Aarhus N, Denmark
[2]Department of Animal Science, AU Foulum, Aarhus University, Blichers Allé 20, DK-8830 Tjele, Denmark

ABSTRACT

Variations in major milk minerals, proteins, and their posttranslational modifications are largely under genetic influence, whereas the effect of nongenetic factors is less studied. Through a controlled feeding experiment (incomplete balanced Latin square design), the effect of concentrate mixtures, based on fava beans, rapeseed meal, or soybean meal as main P and protein sources, on milk composition was examined under typical Danish management conditions. Concentrations of P, Ca, and Mg, together with proteomics for relative quantification of major milk proteins and their isoforms, were analyzed in milk samples from 24 cows sampled in 4 periods. Each cow was fed 1 of the 3 diets in each period with or without addition of exogenous phytase. Cows were blocked by lactation stage into early and mid-lactation (23.3 ± 6.7 and 176 ± 15 d in milk, respectively, at the beginning of the experiment, mean ± standard deviation). Significant effects of feed concentrate mixture were observed for milk protein concentration, milk urea nitrogen, citrate, and the percentage of mixed and preformed fatty acids as well as mineral composition, and their distributions within micellar or serum phases. Furthermore, relative contents of α_{S1}-casein (CN) 9P form and unglycosylated κ-CN and thereby phosphorylation degree of α_{S1}-CN (PD) and the glycosylation degree of κ-CN were found to be significantly affected by these diets. To our knowledge, we are the first to document that feed concentrate mixture can affect the relative concentrations of α_{S1}-CN phosphorylation isoforms in milk, and the results suggested an effect on α_{S1}-CN 9P and PD, but not on α_{S1}-CN 8P. Furthermore, although only significant for α_{S1}-CN 8P, we found a lower relative concentration of α_{S1}-CN 8P and higher α_{S1}-CN 9P (and thus higher PD) in milk from cows in mid compared with early lactation. Also, protein concentration and concentration of Mg in skim milk and serum as well as relative concentration of α-lactalbumin were found to be significantly affected by lactation stage. Addition of dietary exogenous phytase only had a minor effect on milk composition or functionality with significant effect detected for α-lactalbumin and micellar Mg concentration.

Key words: rapeseed, soybean, fava bean, faba bean, ruminant

INTRODUCTION

New regulations require a reduction of nutrient losses and better monitoring of P from farms, as this can cause eutrophication of water environments. Furthermore, predicted future scarcity of P sources (Cordell and White, 2015) and increased demand for sustainable protein (Swensson et al., 2017) have placed pressure on agricultural P and N efficiency. Together with increasing focus on genetically modified organism (**GMO**)-free milk production, these are the main drivers for changes in protein supplementation and have pushed for substitution of imported soybean meal with locally grown protein-rich crops and legumes such as rapeseed meal and fava beans in dairy production (Lehuger et al., 2009; Tufarelli et al., 2012). Due to the restrictive regulations on farm P balance, supply of inorganic P from mineral supplementation in cattle in Denmark is limited, and P supply is mainly through P from feed. Protein-rich feedstuffs usually have high P contents, which results in P overfeeding and high P excretion from cattle (Wu et al., 2000; Nordqvist et al., 2014). Dietary P is mainly stored in the seeds of the plants as phytate, which can only be released by activity of the enzyme, phytase. Rumen microbes are able to degrade most phytate from the feed, but the degree of release can vary depending on passage rate and feed treatment (Humer and Zebeli, 2015). In dairy cows, phytate P is only a small fraction of total P excreted; however, both

Received December 22, 2020.
Accepted April 20, 2021.
*Corresponding author: Nina.Poulsen@food.au.dk

Jarrett et al. (2014) and Brask-Pedersen et al. (2013) found that degradation of phytate in the rumen can be enhanced by addition of exogenous phytase. Giagnoni et al. (2021) found indications that when cows are fed slightly below P recommendations, feeding exogenous phytase can to some extent increase P utilization and balance. From a nutritional perspective, feeding of cows as close as possible to recommended dietary P is the best way to reduce P run-off to the environment. With increasing focus on GMO-free milk production and rapeseed meal being high in P, fava bean meal can be a preferred alternative to soybean meal.

From a milk perspective, changes in P supplementation and accessibility may affect milk P content as well as the distribution between organic and inorganic P fractions in milk. Apart from effects on the nutritional value of minerals in milk, such changes would mainly relate to the dynamic equilibria of calcium-phosphate between CN micellar and serum fractions, of importance for CN micelle structure and stability (Dalgleish and Corredig, 2012). Total P in bovine milk is around 930 to 1,000 mg/L (Lucey and Horne, 2009). In skim milk, organic P refers to the phosphorylated serine residues in the CN polypeptide chains, whereas inorganic P can be divided into 2 phases, a micellar and a serum fraction. Of total inorganic P (20.9 m*M*), the serum fraction constitutes around 54% (11.2 m*M*), whereas the remaining 46% is inorganic P related to calcium-phosphate nanoclusters in the micelles (Gaucheron, 2005). However, when referring to micellar P this includes both P as phosphoserines and calcium-phosphate nanoclusters. With respect to Ca in bovine milk (30 m*M* in total), approximately two-thirds are bound in the CN micelles (20 m*M*) and one-third is in the serum fraction (10 m*M*). In milk serum, it is present either as complexed, mainly with citrate, or as free ionic Ca^{2+} (<10%; Gaucheron, 2005; Lewis, 2011). Magnesium is another important cation in milk. Of total Mg (5.1 m*M*), approximately 35% is bound in the CN micelles (1.8 m*M*), whereas 65% is found in the serum fraction (3.3 m*M*; Gaucheron, 2005). Strong correlations therefore exist between total milk P and protein content, but also between total protein and total Ca and Mg, respectively, in milk and especially between the micellar contents of P, Ca, and Mg (Bijl et al., 2013). This suggests that these minerals are all strongly associated with CN micelles and play a role for CN micelle stability.

Posttranslational modifications (**PTM**) of the CN include phosphorylations (all CN) and glycosylations (κ-CN). Phosphorylation of mainly Ser residues in the CN and glycosylation at Thr residues in the κ-CN sequence occur in the Golgi apparatus and are catalyzed by specific kinases. Phosphorylation and glycosylation of CN are very important features for CN micelle size and stability through interactions with the calcium-phosphate nanoclusters and negative charge from neuraminic acid groups on κ-CN (Holland, 2009). The number of phosphorylations is highest in α_{S2}-, α_{S1}-, and β-CN, and lowest in κ-CN (Holland, 2009). Earlier detailed investigations of major and minor phosphorylation isoforms of α_{S1}-CN and α_{S2}-CN in milk from Montbéliarde cows revealed presence of 9 to 15 phosphorylation sites in α_{S2}-CN, together with the commonly reported α_{S1}-CN 8P and 9P (Fang et al., 2016). Phenotypic correlations and hierarchical clustering suggested that the relative concentrations of phosphorylation isoforms were controlled by different phosphorylation pathways, with one system affecting the lower phosphorylation degrees and another affecting the higher phosphorylation isoforms of both α_{S1}- and α_{S2}-CN (Fang et al., 2016). Heritability estimates for α_{S1}-CN 8P and 9P isoforms were found to be moderate to high in Dutch Holstein (Bijl et al., 2014), and somewhat lower for especially α_{S1}-CN 8P in Danish Holstein (Buitenhuis et al., 2016). Breed differences have also documented higher proportions of the more phosphorylated forms of α_{S1}-CN (9P) and α_{S2}-CN (12P), and a lower proportion of unglycosylated (**UG**) κ-CN in Danish Holstein compared with Danish Jersey (Poulsen et al., 2016). This resulted in higher phosphorylation degrees (**PD**) of α_{S1}- and α_{S2}-CN (defined as α_{S1}-CN 9P/total α_{S1}-CN and α_{S2}-CN 12P/total α_{S2}-CN, respectively), as well as a higher glycosylation degree [**GD**, defined as glycosylated (**G**) κ-CN/total κ-CN] in Danish Holstein compared with Danish Jersey (Poulsen et al., 2016). Days in milk, days before next calving, and also parity further seemed to play a role for variations in the major milk proteins and their specific isoforms (Poulsen et al., 2016; Maciel et al., 2017). Thus, whereas genetics and lactation patterns have been documented to influence different isoforms of the proteins in milk, less is known about the ability to change these isoforms through management and feeding.

In the current study, the aim was to evaluate possible effects of dietary changes on milk P, Ca, and Mg composition and quality, including shares of micellar and serum fractions of these minerals, overall milk composition, fatty acid composition, and protein profiling.

MATERIALS AND METHODS

Experimental Design and Feeding

Samples in this study derive from the feeding experiment reported by Giagnoni et al. (2021). Briefly, 24 Danish Holstein cows, 6 primiparous and 6 multiparous in early lactation (**EL**) and 12 multiparous in mid-

lactation (**ML**), were divided into 4 blocks according to parity and DIM. The cows were randomly assigned to 1 of 6 diets in a 6 × 4 incomplete Latin square design with 4 periods, which resulted in 16 observations per diet. At the beginning of the experiment, EL cows were 23.3 ± 6.7 (mean ± SD) DIM and ML cows were 176 ± 15 DIM. Each period lasted 21 d, of which the first 17 were used for adaptation and the last 4 d for sample collection. In the diets, the concentrate mixture (CM, 40% on DM basis) were based on fava beans (**FAV**), rapeseed meal (**RSM**), or soybean meal (**SBM**) as the main protein source. Each CM was fed with (+, 6,000 FTU/kg of DM) and without (−) the addition of exogenous phytase (EC 3.1.3.8; Novozymes, Denmark), resulting in a 3 × 2 factorial arrangement of diets (**FAV−**, **FAV+**, **RSM−**, **RSM+**, **SBM−**, **SBM+**). Forage constituted 60% on DM basis (2/3 corn silage and 1/3 grass-clover silage) in all diets. The 6 diets were formulated to be below actual P recommendations, with the main protein source supplying 35 to 40% of total P in the diet. The diets were formulated to hold a P concentration of 3.2, 3.4, and 3.2 g/kg of DM and CP concentration of 174, 175, and 179 g/kg of DM in FAV, RSM, and SBM diets, respectively. Diets were mixed daily and fed ad libitum as TMR. Cows were housed in a loose housing system and were assigned to individual feed troughs.

Milk Sampling and Overall Milk Composition

The cows were milked daily at 0600 and 1700 h in a milking parlor, where milk yield was recorded at each individual milking. From each cow, subsamples of milk were taken from the milking on d 18 afternoon and on d 19 morning. Immediately after morning milking, a representative sample was prepared by mixing the afternoon and the morning milk samples, and this was used for further milk analyses. After mixing, fresh full milk samples were analyzed for fat, protein, lactose, urea, and citrate by infrared spectroscopy (Milkoscan FT2+, FOSS Analytical). The instrument was routinely calibrated according to these milk parameters by use of calibration samples provided by Eurofins Scientific. Skim milk samples were prepared by centrifugation at 2,643 × *g* for 30 min at 4°C and subjected to skim milk analysis or frozen for later analysis (−80°C). The cream phase was stored at −20°C until analysis of fatty acid (**FA**) composition using GC as described by Poulsen et al. (2012). For simplicity, the FA are grouped and reported as de novo FA (C4 to C14 including C14:1), mixed origin (C16:0, C16:1, C17:0), and preformed FA (C18:0 and longer), according to Woolpert et al. (2016). Fresh skim milk samples were analyzed for pH (PHM 220 pH meter, RadioMeter) and conductivity (LDM 210 Conductivity meter, RadioMeter). Ethanol stability, defined as the highest concentration of ethanol solution added to milk while not causing any visual coagulation was determined in skim milk, as outlined in Akkerman et al. (2019). High-speed centrifugation was performed on skim milk samples to separate the nonsedimentable (serum) and the sedimentable (micellar) phases. The separation was performed at 100,000 × *g* for 1 h at 21°C using an Optima L-80XP Ultracentrifuge (Beckman Coulter Inc., Brea, CA).

Protein and Mineral Determination

For determination of P, Ca, and Mg concentrations, skim milk and serum samples were acidified in nitric acid and destructed at 1,500 W at 230°C for 25 min in a microwave digestion system (Ultra Wave, single reaction chamber, Milestone). Destructed milk samples were analyzed for P, Ca, and Mg with appropriate colorimetric method according to standard procedures (Siemens Diagnostics R Clinical Methods). Analyses were performed using an auto-analyzer, ADVIA 1800 R Chemistry System (Siemens Medical Solutions). Micellar P, Ca, and Mg were calculated by subtracting the concentration in serum from the total skim milk concentration.

Variations in the composition of the major proteins in skim milk were determined using a reversed-phase liquid chromatography method, where major proteins and specific PTM isoforms of selected proteins were identified with the use of electrospray ionization-MS. Proteins were separated by reversed-phase HPLC using an HPLC 1100 system (Agilent Technologies) with a Jupiter C4 column (250 mm × 2 mm, 5 μm particle size, 300 Å pores; Phenomenex) operated at 40°C and a G1315A diode-array detector with UV detection at 214 nm coupled to a mass selective detector. The liquid chromatography electrospray ionization-MS method used in the present study was outlined by Jensen et al. (2012). Based on UV absorbance profile at 214 nm, relative protein concentrations per skim milk sample were determined for all major CN and whey proteins, together with specific PTM forms using integrated peak areas relative to total peak area of identified proteins per run using the ChemStation software (Agilent Technologies). Identified components included G κ-CN and UG κ-CN, as well as α_{S1}-CN 8P and α_{S1}-CN 9P isoforms together with calculated GD and PD. Each milk sample was analyzed once.

Statistical Analysis

Data were analyzed with R 3.6.3 (R Core Team, 2019; https://www.r-project.org/), using the lmer func-

tion from the lme4 package (Bates et al., 2015), with the following model:

$$Y_{ijklmn} = \mu + CM_i + EN_j + LS_k + PA_l + PE_m + (CMEN)_{ij} + (CMLS)_{ik} + (ENLS)_{jk} + (CMENLS)_{ijk} + C_n + e_{ijklmn},$$

where Y_{ijklmn} is the dependent response variable (n = 96), μ is the overall mean, CM is the fixed effect of concentrate mixtures with different main protein sources (i = FAV, RSM, SBM), EN is the fixed effect of phytase (j = −, +), LS is the fixed effect of lactation stage (k = EL, ML), PA is the fixed effect of parity (l = primiparous, multiparous), PE is the fixed effect of period (m = 1 to 4), $(CMEN)_{ij}$ is the interaction between concentrate mixture and phytase, $(CMLS)_{ik}$ is the interaction between concentrate mixture and lactation stage, $(ENLS)_{jk}$ is the interaction between phytase and lactation stage, $(CMENLS)_{ijk}$ is the interaction between concentrate mixture, phytase, and lactation stage, C is the random effect of cow (n = 1 to 27, as 3 cows were substituted during the experiment), and e_{ijklmn} is the random residual error assumed to be independent with constant variance and normally distributed. The remaining 2-way interactions were tested as well, but none were significant or improved the model. Least squares means and standard error of mean obtained using the *emmeans* package (Lenth, 2019) are presented in the tables, whereas the least squares means reported in text are obtained considering the main effect or interaction which is addressed. Pairwise comparisons were conducted by Tukey post hoc test. Effects with $P < 0.10$ are considered as tendencies and $P < 0.05$ are considered as significant.

Calculation of Pearson correlation values were conducted in R. Pearson correlation figures and their significances ($\alpha = 0.01$) were generated using the *corrplot* R-package (Wei and Simko, 2017).

RESULTS

Overall Milk Composition

Table 1 outlines the effects of CM, phytase addition, lactation stage, and their interactions for overall milk composition, groups of FA, and ethanol stability. Cows fed FAV had lower milk protein concentration compared with cows fed SBM or RSM (3.62 vs. 3.74 or 3.71%, $P < 0.01$ and $P = 0.01$, respectively), whereas milk protein concentration for cows fed SBM and RSM did not differ ($P = 0.73$). Furthermore, cows in EL had lower milk protein concentration compared with cows in ML (3.54 vs. 3.84%, $P = 0.02$, Table 1). Likewise, cows fed FAV had lower milk urea concentration compared with cows fed SBM or RSM (9.52 vs. 11.85 and 12.32 mg/dL, $P < 0.001$ for both pairwise comparisons), whereas milk urea concentration for cows fed SBM and RSM did not differ ($P = 0.40$). For milk citrate, cows fed SBM had lower concentrations compared with cows fed RSM or FAV (0.142 vs. 0.153 and 0.159%, $P = 0.01$ and $P < 0.001$, respectively), whereas milk citrate for cows fed FAV and RSM did not differ ($P = 0.15$). Percentage of mixed fatty acids in total fat was lower in cows fed RSM compared with SBM or FAV (39.8 vs. 41.2 and 41.5%, $P < 0.001$ for both pairwise comparisons), whereas mixed fatty acids for cows fed FAV and SBM did not differ ($P = 0.70$). The percentage of preformed fatty acids was higher in RSM compared with FAV or SBM (27.7 vs. 26.1 and 26.2%, $P < 0.001$ for both pairwise comparisons), whereas preformed fatty acids for cows fed FAV and SBM did not differ ($P = 0.98$). For fat and lactose concentrations together with percentage of de novo FA and ethanol stability, no significant effects were observed.

Milk Minerals

Table 2 presents the effects of the treatments on total, serum, and micellar concentrations of P, Ca, and Mg in skim milk as well as share of micellar P, Ca, and Mg (micellar/total) in skim milk. The CM affected total P concentration in skim milk ($P < 0.01$, Table 2) as cows fed SBM had higher total P concentration in skim milk compared with cows fed FAV or RSM (1.11 vs. 1.08 and 1.08 g/kg). The same pattern was observed for serum P concentration ($P = 0.05$), whereas no significant effect of CM was observed for micellar P concentration ($P = 0.12$). Skim milk P concentration tended to be higher in cows fed diets without addition of phytase (1.10 g/kg) than cows fed diets with addition of phytase (1.08 g/kg; $P = 0.09$). Cows fed FAV had higher serum Ca concentration compared with cows fed RSM or SBM (0.447, 0.433, 0.421 g/kg, $P = 0.02$ and $P < 0.001$, respectively). Furthermore, cows fed RSM had higher milk serum Ca than cows fed SBM ($P = 0.03$). For micellar Ca, cows fed SBM had higher concentration compared with cows fed RSM (0.979 vs. 0.934 g/kg; $P = 0.02$), whereas micellar Ca for cows fed FAV (0.952 g/kg) did not differ from the 2 other feedings. This was also reflected in a higher proportion of micellar Ca for cows fed SBM, compared with cows fed FAV or RSM (69.7 vs. 67.9 and 68.2%, $P < 0.001$ for both pairwise comparisons). Type of CM had limited influence on Mg concentrations in milk, and the only significant effect was on serum Mg ($P = 0.03$), where cows fed SBM had lower serum Mg compared with cows fed FAV (0.055 vs. 0.058 g/kg, $P = 0.02$), whereas cows fed RSM had

Table 1. Full milk composition together with groups of fatty acids (FA) and ethanol stability at different lactation stages provided 3 different concentrate mixtures with or without phytase[1]

		Diet[4]							*P*-value[5]					
Trait[2]	LS[3]	FAV−	FAV+	RSM−	RSM+	SBM−	SBM+	SEM	CM	EN	LS	CM × EN	CM × LS	EN × LS
Fat (g/100 g)	EL	4.50	4.31	4.19	4.24	4.00	4.45	0.225	0.22	0.41	0.62	0.09	0.53	0.95
	ML	4.36	4.16	4.21	4.30	3.77	4.15	0.264						
Protein (g/100 g)	EL	3.50	3.43	3.62	3.54	3.60	3.57	0.083	<0.01	0.36	0.02	0.79	0.76	0.21
	ML	3.75	3.8	3.85	3.84	3.90	3.89	0.107						
Lactose (g/100 g)	EL	4.80	4.70	4.82	4.82	4.83	4.80	0.034	0.57	0.15	0.44	0.60	0.09	0.50
	ML	4.78	4.78	4.78	4.72	4.79	4.77	0.044						
MUN (mg/dL)	EL	9.91	9.71	12.21	12.27	12.51	12.74	0.664	<0.001	0.73	0.42	0.91	0.07	0.66
	ML	9.25	9.20	12.69	12.10	11.11	11.03	0.804						
Citric acid (g/100 g)	EL	0.169	0.159	0.158	0.152	0.146	0.144	0.007	<0.001	0.54	0.50	0.81	0.79	0.11
	ML	0.153	0.156	0.149	0.151	0.137	0.141	0.009						
De novo FA (%)	EL	32.7	32.3	32.6	32.8	32.8	33.0	0.57	0.64	0.75	0.57	0.95	0.81	0.78
	ML	32.2	32.3	32.3	32.2	32.6	32.1	0.72						
Mixed FA (%)	EL	40.6	41.7	39.8	39.9	41.0	40.1	0.83	<0.001	0.81	0.60	0.85	0.19	0.86
	ML	42.1	41.5	39.8	39.9	41.6	42.1	1.05						
Preformed FA (%)	EL	26.6	26.0	27.6	27.3	26.2	26.7	0.70	<0.001	0.97	0.85	0.86	0.27	0.68
	ML	25.7	26.2	28.0	28.0	25.9	25.8	0.85						
Ethanol stability (%)	EL	88.4	87.6	88.3	89.8	87.0	84.0	2.12	0.15	0.83	0.53	0.23	0.45	0.55
	ML	86.2	85.5	85.0	87.5	85.8	85.1	2.58						

[1]Least squares means and *P*-values are reported for the specific effects and their interactions.

[2]Fatty acids are reported as % wt/wt of total fat, and ethanol stability as the highest concentration of ethanol solution added to milk not causing any visual coagulation.

[3]Lactation stage: EL = early lactation (23.3 ± 6.7 DIM, mean ± SD), ML = mid-lactation (176 ± 15 DIM, mean ± SD).

[4]FAV = fava bean diet, RSM = rapeseed meal diet, and SBM = soybean meal diet with (+) or without (−) addition of phytase.

[5]CM = concentrate mixture (FAV, RSM, and SBM), EN = phytase (+ and −), and LS = lactation stage (EL and ML).

serum Mg somewhere in between (0.056 g/kg, $P > 0.05$ for both pairwise comparisons). However, a significant effect of lactation stage was observed for skim milk ($P < 0.01$) and serum Mg ($P = 0.02$), and a significant effect of addition of phytase was observed for micellar Mg ($P = 0.04$) and proportion of micellar Mg ($P = 0.05$). Cows in EL had lower skim milk Mg and serum Mg compared with cows in ML (0.082 vs. 0.094, $P < 0.01$, and 0.052 vs. 0.060 g/kg, $P = 0.02$, respectively). Addition of phytase to the diets resulted in lower concentration of micellar Mg in milk compared with milk from cows fed without addition of phytase (0.030 vs. 0.032 g/kg, $P = 0.04$). This was also reflected in the proportion of micellar Mg, where phytase addition resulted in a lower fraction of micellar Mg compared with milk from cows fed without phytase (34.6 vs. 36.7%, $P = 0.05$). In addition, significant interaction effects were observed between lactation stage and CM for micellar Mg ($P < 0.01$) and proportion of micellar Mg ($P = 0.02$). No significant effect was observed on ionic Ca concentrations.

Protein Distribution

Significant effects of CM were observed for proportions of α_{S1}-CN 9P and UG κ-CN concentrations, as well as for PD and GD (Table 3). Cows fed FAV had a higher proportion of α_{S1}-CN 9P compared with cows fed RSM or SBM (6.96% vs. 6.52% and 6.46% as a proportion of total protein, $P < 0.001$ for both pairwise comparisons), whereas α_{S1}-CN 9P for cows fed RSM and SBM did not differ ($P = 0.87$). This was also reflected in a higher PD for cows fed FAV compared with cows fed RSM or SBM (21.8% vs. 20.6% and 20.5%, $P < 0.01$ and $P < 0.001$, respectively, Figure 1A). The PD for cows fed RSM and SBM did not differ ($P = 0.89$). The CM affected the proportion of UG κ-CN ($P < 0.001$), as cows fed FAV had a lower relative proportion than cows fed RSM or SBM (5.84% vs. 6.03% and 6.22%, respectively), as reflected in an effect of CM on GD ($P = 0.01$, Figure 1B), where a higher GD was observed for cows fed FAV compared with SBM (37.9% vs. 36.0% and 35.3%, respectively). Both phytase addition and lactation stage affected α-LA, with higher proportions of α-LA in EL compared with ML (3.19 vs. 2.70%, $P = 0.02$) and lower α-LA proportions by addition of dietary phytase (2.86 vs. 3.03%, as proportion of total protein, $P = 0.02$). Furthermore, there was a significant effect of lactation stage on α_{S1}-CN 8P, with a higher proportion in EL compared with ML (25.8 vs. 24.4% as a proportion of total protein, $P = 0.04$). In addition, an interaction effect was observed for β-LG between phytase and lactation stage ($P < 0.01$), as β-LG proportion decreased in EL cows, when phytase was added to the diet, whereas it increased in ML cows.

Correlations Among Milk Mineral and Protein Traits

Several milk traits were significantly correlated (Figure 2, correlations marked with a cross were not significant based on the F-test ($P > 0.01$)). Apart from significant correlations among the minerals fractions, mineral concentrations in skim milk and serum were highly positively correlated with protein concentration (r >0.6). Furthermore, the relative proportion of β-LG and protein concentration was positively correlated (r = 0.44), whereas both α_{S1}-CN 9P (r = −0.33) and PD (r = −0.27) had weak but significant negative correlations to protein concentration. Urea concentration was weakly negatively correlated with Mg in skim milk and serum as well as with β-LG. Mineral traits were highly positively correlated among one another and within each mineral in the skim milk and micellar phase. Furthermore, weak positive correlations existed between β-LG and both Ca and Mg in skim milk, Mg in serum and Ca in the micellar phase. A strong positive significant correlation between α_{S1}-CN 9P and PD (r = 0.97) was observed, and both traits had strong negative significant correlations with α_{S1}-CN 8P (r = −0.69 and r = −0.84, respectively). Further, α_{S1}-CN 8P had a strong correlation with total α_{S1}-CN (r = 0.72). Furthermore, P in serum and P in skim milk were positively correlated with α_{S1}-CN 8P (r = 0.37 for both) and negatively correlated with α_{S1}-CN 9P (r = −0.39 for both) and PD (r = −0.42 for both), respectively. Different shares of κ-CN were also significantly correlated with minerals and other protein fractions. The G κ-CN had a weak but significant positive correlation to Ca in serum (r = 0.29), whereas UG k-CN had a negative correlation to Ca in serum (r = −0.29) and a positive correlation to P in serum (r = 0.30). These associations were further reflected in a significant correlation with GD. Interestingly, G k-CN was significantly negatively correlated with α_{S1}-CN 8P (r = −0.51), but positively correlated with α_{S1}-CN 9P (r = 0.50), which in turn is also reflected in significant correlations among the α_{S1}-CN phosphoforms and GD, as well as between GD and PD (r = 0.63).

DISCUSSION

Using a randomized incomplete Latin square design, we have analyzed the effect of CM, exogenous phytase addition, lactation stage, and their interactions on milk composition traits. Attempts to affect P utilization and excretion by phytase resulted in modest effects for P

Table 2. Skim milk, serum, and micellar concentrations of P, Ca, and Mg (g/kg) and micellar percentage of these minerals at different lactation stages provided 3 different concentrate mixtures with or without phytase[1]

		Diet[4]							*P*-value[5]					
Trait[2]	LS[3]	FAV−	FAV+	RSM−	RSM+	SBM−	SBM+	SEM	CM	EN	LS	CM × EN	CM × LS	EN × LS
Skim milk P (g/kg)	EL	1.06	1.03	1.09	1.06	1.09	1.10	0.028	0.05	0.09	0.40	0.93	0.15	0.96
	ML	1.11	1.11	1.09	1.08	1.14	1.10	0.035						
Skim milk Ca (g/kg)	EL	1.38	1.35	1.38	1.35	1.37	1.39	0.037	0.07	0.12	0.43	0.36	0.10	0.52
	ML	1.47	1.42	1.39	1.34	1.42	1.42	0.046						
Skim milk Mg (g/kg)	EL	0.085	0.078	0.084	0.081	0.081	0.080	0.0029	0.16	0.07	<0.01	0.69	0.22	0.11
	ML	0.096	0.096	0.092	0.092	0.093	0.092	0.0037						
Serum P (g/kg)	EL	0.497	0.485	0.512	0.493	0.512	0.513	0.015	0.05	0.29	0.74	0.32	0.68	0.43
	ML	0.480	0.502	0.491	0.495	0.516	0.486	0.019						
Serum Ca (g/kg)	EL	0.453	0.448	0.445	0.434	0.425	0.429	0.011	<0.001	0.72	0.52	0.30	0.88	0.20
	ML	0.441	0.444	0.426	0.429	0.408	0.423	0.014						
Serum Mg (g/kg)	EL	0.056	0.054	0.050	0.053	0.051	0.050	0.0023	0.03	0.66	0.02	0.61	0.37	0.48
	ML	0.059	0.063	0.061	0.061	0.059	0.058	0.0029						
Micellar P (g/kg)	EL	0.567	0.549	0.581	0.565	0.579	0.585	0.020	0.12	0.14	0.16	0.69	0.14	0.73
	ML	0.624	0.608	0.601	0.584	0.626	0.616	0.025						
Micellar Ca (g/kg)	EL	0.927	0.905	0.940	0.915	0.945	0.960	0.034	0.03	0.16	0.32	0.74	0.18	0.51
	ML	0.998	0.980	0.961	0.921	1.019	0.991	0.043						
Micellar Mg (g/kg)	EL	0.030	0.025	0.033	0.028	0.030	0.030	0.0020	0.55	0.04	0.07	0.31	<0.01	0.25
	ML	0.036	0.033	0.031	0.032	0.035	0.034	0.0023						
Micellar P (%)	EL	53.3	53.1	53.3	53.6	53.1	53.3	0.99	0.51	0.35	0.16	0.13	0.21	0.24
	ML	57.0	54.8	55.2	53.9	54.8	55.9	1.26						
Micellar Ca (%)	EL	67.2	66.6	67.8	67.8	68.9	69.0	1.01	<0.001	0.16	0.30	0.99	0.28	0.29
	ML	69.2	68.8	69.2	68.0	71.2	69.9	1.28						
Micellar Mg (%)	EL	34.8	31.2	40.1	34.7	36.6	37.4	1.96	0.17	0.05	0.89	0.27	0.02	0.48
	ML	38.4	34.3	33.2	33.7	37.1	36.7	2.29						
Ionic Ca^{2+} (m*M*)	EL	1.88	1.99	1.99	1.93	1.92	2.04	0.072	0.64	0.14	0.65	0.33	0.90	0.87
	ML	1.91	1.89	1.91	1.96	1.86	1.98	0.087						

[1]Least squares means and *P*-values reported for the specific effects and their interactions.

[2]Concentration (g/kg) of minerals in skim milk as well as concentrations in the micellar and serum phases of skim milk. Micellar P, Ca, or Mg % refers to the proportion of micellar P, Ca, or Mg relative to total skim milk concentrations. Ionic calcium is given in m*M*.

[3]Lactation stage: EL = early lactation (23.3 ± 6.7 DIM, mean ± SD), ML = mid-lactation (176 ± 15 DIM, mean ± SD).

[4]FAV = fava bean diet, RSM = rapeseed meal diet, and SBM = soybean meal diet with (+) or without (−) addition of phytase.

[5]CM = concentrate mixture (FAV, RSM, and SBM), EN = phytase (+ and −), and LS = lactation stage (EL and ML).

Table 3. Protein proportions as a percentage of total protein together with phosphorylation (PD) and glycosylation (GD) degrees at different lactation stages provided 3 different concentrate mixtures with or without phytase[1]

		Diet[4]							*P*-value[5]					
Trait[2]	LS[3]	FAV−	FAV+	RSM−	RSM+	SBM−	SBM+	SEM	CM	EN	LS	CM × EN	CM × LS	EN × LS
α_{S1}-CN (%)	EL	31.9	32.3	31.7	32.3	32.0	32.0	0.45	0.36	0.60	0.26	0.95	0.63	0.30
	ML	31.9	31.6	31.3	31.2	31.2	31.3	0.54						
α_{S1}-CN 8P (%)	EL	25.4	25.5	25.9	26.0	25.9	25.8	0.48	0.70	0.91	0.04	0.99	0.23	0.92
	ML	24.5	24.5	24.4	24.2	24.5	24.6	0.60						
α_{S1}-CN 9P (%)	EL	6.5	6.8	5.9	6.2	6.0	6.2	0.33	<0.001	0.34	0.13	0.85	0.26	0.09
	ML	7.3	7.2	7.0	7.0	6.9	6.8	0.42						
PD (%)	EL	20.5	21.2	18.4	19.4	18.9	19.3	0.99	<0.001	0.34	0.08	0.73	0.12	0.16
	ML	22.9	22.7	22.3	22.5	22.0	21.6	1.27						
α_{S2}-CN (%)	EL	7.8	7.9	8.0	7.9	8.1	8.3	0.34	0.25	0.27	0.29	0.53	0.15	0.66
	ML	7.1	7.5	7.4	7.9	7.5	7.2	0.43						
β-CN (%)	EL	37.1	37.7	36.9	37.1	36.9	37.2	0.54	0.33	0.96	0.82	0.52	0.82	0.09
	ML	37.2	37.0	37.5	36.6	36.7	36.8	0.68						
κ-CN (%)	EL	9.2	9.2	9.3	9.4	9.7	9.8	0.39	0.50	0.62	0.76	0.78	0.14	0.85
	ML	9.8	9.6	9.5	9.6	9.5	9.6	0.49						
G κ-CN (%)	EL	3.5	3.4	3.2	3.4	3.4	3.4	0.29	0.22	0.61	0.63	0.41	0.54	0.94
	ML	3.9	3.7	3.5	3.6	3.3	3.6	0.37						
UG κ-CN (%)	EL	5.7	5.9	6.1	6.0	6.3	6.4	0.20	<0.001	0.83	0.92	0.81	0.63	0.47
	ML	5.9	5.9	6.0	6.1	6.2	6.0	0.26						
GD (%)	EL	37.6	36.3	34.4	36.0	34.6	34.6	1.93	0.01	0.59	0.53	0.39	0.97	0.71
	ML	39.1	38.5	36.9	36.8	34.8	37.4	2.44						
α-LA (%)	EL	3.3	3.0	3.4	3.1	3.3	3.1	0.16	0.89	0.02	0.02	0.99	0.40	0.46
	ML	2.8	2.7	2.7	2.6	2.8	2.7	0.20						
β-LG (%)	EL	7.9	7.0	8.0	7.5	7.7	6.7	0.48	0.77	0.07	0.17	0.30	0.08	<0.01
	ML	8.6	8.4	8.0	8.5	8.4	8.8	0.61						

[1]Least squares means and *P*-values are reported for the specific effects and their interactions.

[2]Milk protein composition % wt/wt of total protein in milk. G κ-CN =glycosylated κ-CN; UG κ-CN = unglycosylated κ-CN. Phosphorylation degree (PD) % = α_{S1}-CN 9P/total α_{S1}-CN. Glycosylation degree (GD) % = G κ-CN/total κ-CN.

[3]Lactation stage: EL = early lactation (23.3 ± 6.7 DIM, mean ± SD), ML = mid-lactation (176 ± 15 DIM, mean ± SD).

[4]FAV = fava bean diet, RSM = rapeseed meal diet, and SBM soybean meal diet with (+) or without (−) addition of phytase.

[5]CM = concentrate mixture (FAV, RSM, and SBM), EN = phytase (+ and −), and LS = lactation stage (EL and ML).

digestibility in EL, but not in ML cows, and no significant effect of phytase addition was found on overall milk compositional traits (Giagnoni et al., 2021). This was also evident from this study, where only micellar Mg concentration and relative proportion of α-LA in milk were significantly affected by addition of exogenous phytase. In contrast, effects of CM and lactation stage on milk compositional traits were evident.

Milk Composition

Giagnoni et al. (2021) documented significant effect of CM on milk protein concentration, total P, and total Ca, as well as for utilization efficiencies of P and CP

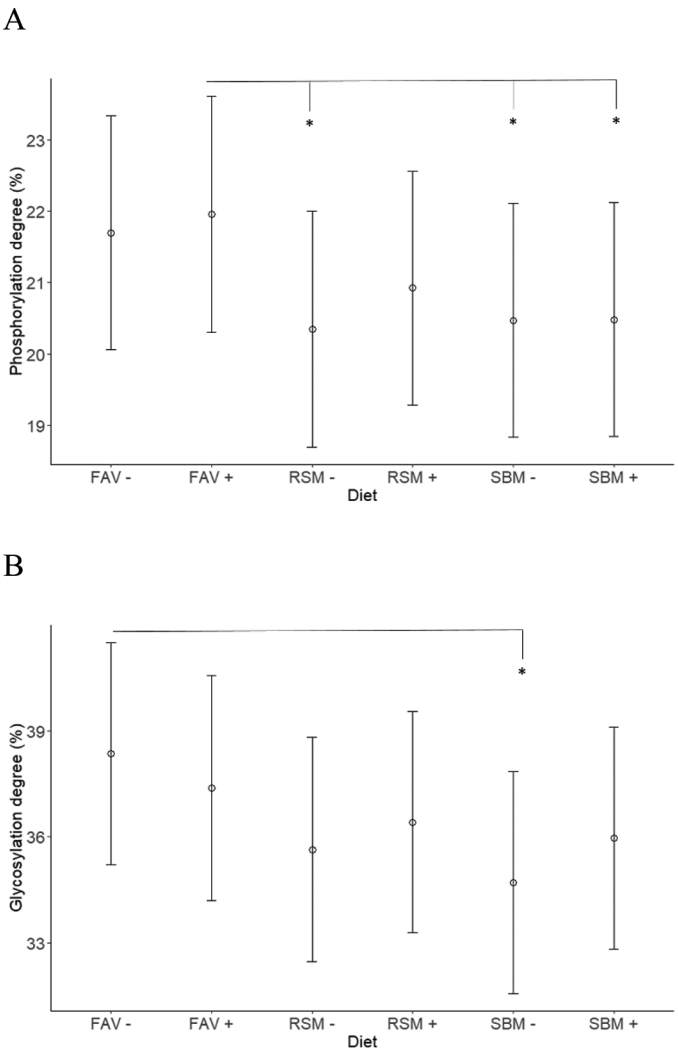

Figure 1. Least squares means (±confidence intervals) for (A) phosphorylation degree (PD) of α_{S1}-CN (%), and (B) glycosylation degree (GD) of κ-CN (%) for cows fed fava beans (FAV), rapeseed meal (RSM), and soybean meal (SBM) diets with (+) or without (−) phytase. Tukey test between diet pairs: $^*P < 0.05$.

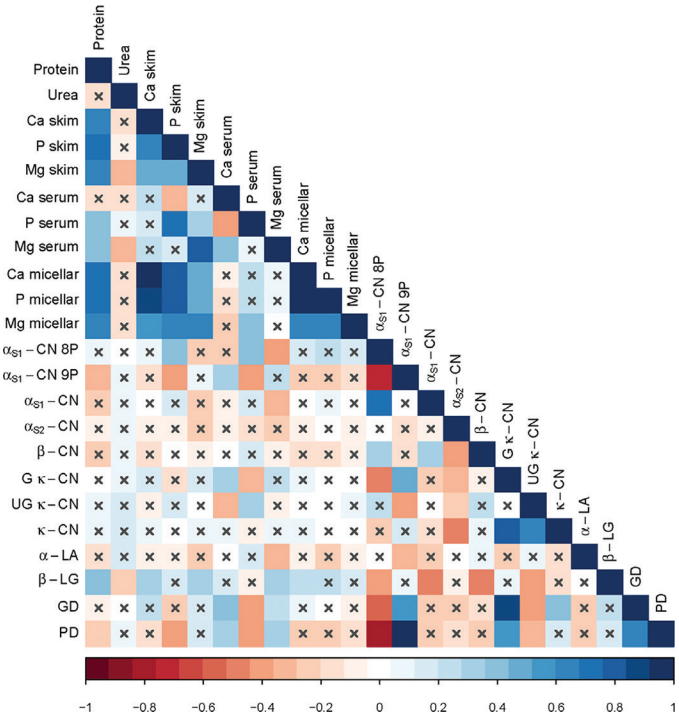

Figure 2. Correlations among pairs of protein, urea, mineral fractions, and protein isoforms. G κ-CN = glycosylated κ-CN; UG κ-CN = unglycosylated κ-CN. Phosphorylation degree (PD) % = α_{S1}-CN 9P/total α_{S1}-CN. Glycosylation degree (GD) % = G κ-CN/total κ-CN. Correlations marked with × were not significant based on the *F*-test ($P > 0.01$).

(calculated as milk output of the nutrient divided by intake of nutrient). Overall milk compositional traits are slightly different in the present study as compared with those reported by Giagnoni et al. (2021) using data from the same feeding experiment. This is because the data presented here, which are based on pooled representative milk samples collected over 2 milkings, were measured on an in-house Milkoscan, whereas the numbers reported by Giagnoni et al. (2021) are based on 6 individual samples sent to an external milk testing laboratory. However, comparable results of protein concentrations are obtained for lactation stage and CM. Higher milk protein concentration in milk from cows fed SBM as compared with FAV and partly RSM may relate to the lower CP intake in cows fed FAV as compared with cows fed RSM and SBM (3.74 vs. 4.06 and 4.05 kg/d; Giagnoni et al., 2021). This may also explain the lower milk urea concentration in cows fed FAV compared with SBM and RSM. Hence, the study clearly documents the relationship between CP in the diet (and intake) on one hand and MUN on the other, as also reported by others (e.g., Baker et al., 1995). Regarding fat, rapeseed is rich in especially C18:1 *cis* FA and soybean in C18:2n-6. This probably drives the

higher proportion of preformed fatty acids in milk from cows fed RSM and SBM compared with FAV. Contrary to this, milk from cows fed FAV was highest in mixed fatty acids. Inclusion of fava beans in diets for dairy cows has previously been associated with increasing C16:0 concentration in milk (Poulsen et al., 2020). We were unable to document higher citrate concentration in cows in EL compared with ML cows, as otherwise reported by Garnsworthy et al. (2006), but this may reflect that the current group of cows was on average 73 DIM during the overall experiment compared with an average of 13 d in lactation for the EL group in Garnsworthy et al. (2006).

Minerals

Total and micellar concentrations of P, Ca, and Mg are generally tightly related to the protein concentration and their concentrations in milk are strongly correlated (Bijl et al., 2013). This was also documented here. However, the distribution between the micellar and serum phase vary considerably among these minerals. Here, on average 54.6, 67.7, and 34.7% of the P, Ca, and Mg, respectively, were present in the micellar phase, which are well in line with the distributions reported by Jensen et al. (2012; 58.6, 66.4, and 30.2% for P, Ca, and Mg). Given the close relation to milk protein concentration, effect of CM on milk protein concentration would be expected to result in higher levels of minerals in cows fed SBM compared with especially FAV. This pattern was also observed for skim milk and serum P (but not for micellar P), as well as micellar Ca and proportion of micellar Ca, and thus lower serum Ca concentrations were found in milk from cows fed SBM and RSM compared with FAV. This documents that changes in diet can affect not only milk protein concentration, but also mineral concentrations and their distributions in the milk phases.

Apart from higher levels of P, Ca, and Mg within the first weeks of lactation and before drying off, the concentration of Mg, Ca, and P is not greatly influenced by stage of lactation (Gaucheron, 2005). Here, significant higher milk Mg concentration was observed in ML compared with EL. Why addition of phytase resulted in lower micellar Mg concentration in milk is not clear, but considering that the effect on phytate P and P digestibility was small (Giagnoni et al., 2021), an effect on P fraction in the milk was not expected.

Protein Composition

Although only significant for α_{S1}-CN 8P, a lower proportion of α_{S1}-CN 8P and a higher proportion of α_{S1}-CN 9P (and higher PD) were observed in milk from cows in ML compared with milk from cows in EL. This is in line with Fang et al. (2017), who determined how phosphorylation isoforms of α_{S1}-CN and α_{S2}-CN were subject to both genetic and nongenetic factors and found effects of both parity and lactation stage on most milk protein phosphorylation isoforms and PD. The study documents increases in the relative concentrations of higher phosphorylation isoform of α_{S1}-CN and α_{S2}-CN and thus accompanied decreases in relative concentrations of the lower phosphorylation isoforms over lactation. Comparable results were found by Poulsen et al. (2016), where α_{S1}-CN isoforms were also significantly affected by parity and lactation stage, whereas these nongenetic factors only had a limited effect on α_{S2}-CN in milk from Danish Holstein cows. However, to our knowledge, we are the first to document that variation in CM can affect α_{S1}-CN phosphorylation isoforms, specifically α_{S1}-CN 9P and PD, whereas an effect on α_{S1}-CN 8P could not be documented. Hence, the underlying mechanisms affecting these forms are again evidently different and relate to both genetic and nongenetic factors. Interestingly, similar effects are observed for κ-CN isoforms, where the relative concentration of UG κ-CN and GD are also significantly affected by CM in the diet.

Among milk compositional traits, significant correlations relate protein concentration with mineral and protein composition. The results on one hand confirm that higher protein concentration is related to higher mineral concentrations, but also show a negative correlation to α_{S1}-CN 9P (and thus PD). So, lower PD seems to be related to higher P in milk, but the underlying mechanisms for this are not clear. Although the genetic influence on PTM variation is documented to be strong (Bijl et al., 2014; Buitenhuis et al., 2016), our results suggest that management factors can also play a significant role.

Changes in the protein supply in dairy production toward more locally grown and GMO-free feed could replace imported soybean meal with fava beans and rapeseed, which are crops suitable for European production systems. Use of FAV instead of SBM decreased protein and mineral concentration in milk. In addition, the lower concentration of P in fava beans compared with rapeseed and soybean meal facilitates feeding P closer to the recommendation. Furthermore, use of FAV increased the relative content of α_{S1}-CN 9P and thereby increased the PD, as well as being associated with lower UG κ-CN and thus higher GD. The underlying mechanisms for increasing PD are not clear, but the results suggest that feeding strategies leading to lower skim milk P also increase PD, which may relate to availability of P in the mammary gland or be a result of genes or pathways responsible for the production of

α_{S1}-CN 9P over α_{S1}-CN 8P. Even subtle differences in PD as observed here have previously been associated with impaired milk coagulation properties (Jensen et al., 2012), but this association is not well understood. Increasing protein, P, Ca, and Mg concentrations are on the other hand associated with improved milk coagulation properties (Tsioulpas et al., 2007; Jensen et al., 2012). These studies document that even subtle differences in milk quality, like those observed here, can manifest into differences in the technological properties of milk.

CONCLUSIONS

Feeding cows with concentrate mixtures based on different main protein sources affected mineral composition in milk and also their distributions between micellar and soluble phases. In addition, the proportions of α_{S1}-CN 9P and UG κ-CN and thereby the phosphorylation degree of α_{S1}-CN (PD) and glycosylation degree of κ-CN (GD) were affected. To our knowledge, we are the first to document dietary changes to differentially affect contents of α_{S1}-CN phosphorylation isoforms, by suggesting an effect on α_{S1}-CN 9P and PD, but not on α_{S1}-CN 8P. Furthermore in relation to lactation, although only significant for α_{S1}-CN 8P, we found a lower relative concentration of α_{S1}-CN 8P and higher α_{S1}-CN 9P (and thus higher PD) in cows in ML compared with cows in EL. Thus, our study supports that underlying mechanisms affecting these isoforms are evidently different and our results suggest that both management and physiological factors can influence PD of the CN.

ACKNOWLEDGMENTS

The study was part of the 2 projects "Reduced excretion of phosphorus in dairy cows" and "Indicators in milk for nitrogen and phosphorus excretion" both financed by the Danish Milk Levy Foundation. The authors thank Gitte Hald Kristiansen (Department of Food Science, Aarhus University, Denmark) for excellent technical assistance; Adam Christian Storm (Novozymes A/S, Bagsværd, Denmark) for providing the phytase enzyme used in the experiment; the barn crew at AU Foulum (Tjele, Denmark) for animal care, feeding, and milking; and Torben Larsen (Department of Animal Science, Aarhus University, Tjele, Denmark) for colorimetric analysis of minerals in milk fractions. The authors have not stated any conflicts of interest.

REFERENCES

Akkerman, M., L. B. Larsen, J. Sørensen, and N. A. Poulsen. 2019. Natural variations of citrate and calcium in milk and their effects on milk processing properties. J. Dairy Sci. 102:6830–6841. https://doi.org/10.3168/jds.2018-16195.

Baker, L. D., J. D. Ferguson, and W. Chalupa. 1995. Responses in urea and true protein of milk to different protein feeding schemes for dairy cows. J. Dairy Sci. 78:2424–2434. https://doi.org/10.3168/jds.S0022-0302(95)76871-0.

Bates, D., M. Mächler, B. M. Bolker, and S. C. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1–48. https://doi.org/10.18637/jss.v067.i01.

Bijl, E., H. van Valenberg, T. Huppertz, and A. van Hooijdonk. 2013. Protein, casein, and micellar salts in milk: Current content and historical perspectives. J. Dairy Sci. 96:5455–5464. https://doi.org/10.3168/jds.2012-6497.

Bijl, E., H. J. F. van Valenberg, T. Huppertz, A. C. M. van Hooijdonk, and H. Bovenhuis. 2014. Phosphorylation of α_{S1}-casein is regulated by different genes. J. Dairy Sci. 97:7240–7246. https://doi.org/10.3168/jds.2014-8061.

Brask-Pedersen, D. N., L. V. Glitsø, L. K. Skov, P. Lund, and J. Sehested. 2013. Effect of exogenous phytase on degradation of inositol phosphate in dairy cows. J. Dairy Sci. 96:1691–1700. https://doi.org/10.3168/jds.2011-5278.

Buitenhuis, B., N. A. Poulsen, G. Gebreyesus, and L. B. Larsen. 2016. Estimation of genetic parameters and detection of chromosomal regions affecting the major milk proteins and their post translational modifications in Danish Holstein and Danish Jersey cattle. BMC Genet. 17:114. https://doi.org/10.1186/s12863-016-0421-2.

Cordell, D., and S. White. 2015. Tracking phosphorus security: Indicators of phosphorus vulnerability in the global food system. Food Secur. 7:337–350. https://doi.org/10.1007/s12571-015-0442-0.

Dalgleish, D. G., and M. Corredig. 2012. The structure of the casein micelle of milk and its changes during processing. Annu. Rev. Food Sci. Technol. 3:449–467. https://doi.org/10.1146/annurev-food-022811-101214.

Fang, Z. H., H. Bovenhuis, A. Delacroix-Buchet, G. Miranda, D. Boichard, M. H. P. W. Visker, and P. Martin. 2017. Genetic and nongenetic factors contributing to differences in αS-casein phosphorylation isoforms and other major milk proteins. J. Dairy Sci. 100:5564–5577. https://doi.org/10.3168/jds.2016-12338.

Fang, Z. H., M. H. P. W. Visker, G. Miranda, A. Delacroix-Buchet, H. Bovenhuis, and P. Martin. 2016. The relationships among bovine α_S-casein phosphorylation isoforms suggest different phosphorylation pathways. J. Dairy Sci. 99:8168–8177. https://doi.org/10.3168/jds.2016-11250.

Garnsworthy, P. C., L. L. Masson, A. L. Lock, and T. T. Mottram. 2006. Variation of milk citrate with stage of lactation and de novo fatty acid synthesis in dairy cows. J. Dairy Sci. 89:1604–1612. https://doi.org/10.3168/jds.S0022-0302(06)72227-5.

Gaucheron, F. 2005. The minerals of milk. Reprod. Nutr. Dev. 45:473–483. https://doi.org/10.1051/rnd:2005030.

Giagnoni, G., P. Lund, J. Sehested, and M. Johansen. 2021. Effect of exogenous dietary phytase and concentrate mixtures based on faba beans, rapeseed meal or soybean meal as main protein source on phytate and total phosphorus excretion in dairy cows. Anim. Feed Sci. Technol. 276:114913. https://doi.org/10.1016/j.anifeedsci.2021.114913.

Holland, J. W. 2009. Post-translational modifications of casein. Pages 107–132 in Milk Proteins: From Expression to Food. A. Thompson, M. Boland, and H. Singh. Academic Press, Elsevier.

Humer, E., and Q. Zebeli. 2015. Phytate in feed ingredients and potentials for improving the utilization of phosphorus in ruminant nutrition. Anim. Feed Sci. Technol. 209:1–15. https://doi.org/10.1016/j.anifeedsci.2015.07.028.

Jarrett, J. P., J. W. Wilson, P. P. Ray, and K. F. Knowlton. 2014. The effects of forage particle length and exogenous phytase inclusion on phosphorus digestion and absorption in lactating cows. J. Dairy Sci. 97:411–418. https://doi.org/10.3168/jds.2013-7124.

Jensen, H. B., N. A. Poulsen, K. K. Andersen, M. Hammershøj, H. D. Poulsen, and L. B. Larsen. 2012. Distinct composition of bovine milk from Jersey and Holstein-Friesian cows with good, poor, or noncoagulation properties as reflected in protein genetic variants

and isoforms. J. Dairy Sci. 95:6905–6917. https://doi.org/10.3168/jds.2012-5675.
Lehuger, S., B. Gabrielle, and N. Gagnaire. 2009. Environmental impact of the substitution of imported soybean meal with locally-produced rapeseed meal in dairy cow feed. J. Clean. Prod. 17:616–624. https://doi.org/10.1016/j.jclepro.2008.10.005.
Lenth, R. 2019. Emmeans: Estimated marginal means, aka least squares means. https://CRAN.R-project.org/package=emmeans.
Lewis, M. J. 2011. The measurement and significance of ionic calcium in milk - A review. Int. J. Dairy Technol. 64:1–13. https://doi.org/10.1111/j.1471-0307.2010.00639.x.
Lucey, J. A., and D. S. Horne. 2009. Milk salts: Technological significance. Pages 351–380 in Advanced Dairy Chemistry: Volume 3: Lactose, Water, Salts and Minor Constituents. 3rd ed. P. L. H. McSweeney and P. F. Fox, ed. Springer Science +Business Media New York.
Maciel, G. D., L. Mogensen, J. O. Lehmann, U. Kidmose, T. Kristensen, L. B. Larsen, and N. A. Poulsen. 2017. Impaired milk quality and cheese making properties is not a concern for managing cows for 15 or 18 months calving intervals. Int. Dairy J. 70:2–11. https://doi.org/10.1016/j.idairyj.2016.12.014.
Nordqvist, M., K. Holtenius, and R. Spörndly. 2014. Methods for assessing phosphorus overfeeding on organic and conventional dairy farms. Animal 8:286–292. https://doi.org/10.1017/S1751731113002103.
Poulsen, N. A., F. Gustavsson, M. Glantz, M. Paulsson, L. B. Larsen, and M. K. Larsen. 2012. The influence of feed and herd on fatty acid composition in 3 dairy breeds (Danish Holstein, Danish Jersey, and Swedish Red). J. Dairy Sci. 95:6362–6371. https://doi.org/10.3168/jds.2012-5820.
Poulsen, N. A., L. Hein, M. Kargo, and A. J. Buitenhuis. 2020. Realization of breeding values for milk fatty acids in relation to seasonal variation in organic milk. J. Dairy Sci. 103:2434–2441. https://doi.org/10.3168/jds.2019-17065.
Poulsen, N. A., H. B. Jensen, and L. B. Larsen. 2016. Factors influencing degree of glycosylation and phosphorylation of caseins in individual cow milk samples. J. Dairy Sci. 99:3325–3333. https://doi.org/10.3168/jds.2015-10226.
Swensson, C., H. Lindmark-Månsson, A. Smedman, M. Henriksson, and A. K. Modin Edman. 2017. Protein efficiency in intensive dairy production: A Swedish example. J. Sci. Food Agric. 97:4890–4897. https://doi.org/10.1002/jsfa.8362.
Tsioulpas, A., A. S. Grandison, and M. J. Lewis. 2007. Changes in physical properties of bovine milk from the colostrum period to early lactation. J. Dairy Sci. 90:5012–5017. https://doi.org/10.3168/jds.2007-0192.
Tufarelli, V., R. U. Khan, and V. Laudadio. 2012. Evaluating the suitability of field beans as a substitute for soybean meal in early-lactating dairy cow: Production and metabolic responses. Anim. Sci. J. 83:136–140. https://doi.org/10.1111/j.1740-0929.2011.00934.x.
Wei, T., and V. Simko. 2017. R package "corrplot": Visualization of a Correlation Matrix (Version 0.84). https://github.com/taiyun/corrplot.
Woolpert, M. E., H. M. Dann, K. W. Cotanch, C. Melilli, L. E. Chase, R. J. Grant, and D. M. Barbano. 2016. Management, nutrition, and lactation performance are related to bulk tank milk de novo fatty acid concentration on northeastern US dairy farms. J. Dairy Sci. 99:8486–8497. https://doi.org/10.3168/jds.2016-10998.
Wu, Z., L. D. Satter, and R. Sojo. 2000. Milk production, reproductive performance, and fecal excretion of phosphorus by dairy cows fed three amounts of phosphorus. J. Dairy Sci. 83:1028–1041. https://doi.org/10.3168/jds.S0022-0302(00)74967-8.

ORCIDS

N. A. Poulsen https://orcid.org/0000-0001-9983-9663
G. Giagnoni https://orcid.org/0000-0001-8153-3041
M. Johansen https://orcid.org/0000-0002-2274-8939
P. Lund https://orcid.org/0000-0002-9113-4500
L. B. Larsen https://orcid.org/0000-0001-9674-0107